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Attorney's Docket No. 035718/260673

PATENT

In The United States Patent And Trademark Office

Appl. No.:	10/617,978
Confirmation No.:	4095
Applicant(s):	Herrmann et al.
Filed:	July 11, 2003
Art Unit:	1638
Examiner:	Anne R. Kubelik
Title:	ISOLATED NUCLEIC ACID MOLECULES ENCODING ORALLY ACTIVE ANDROCTONUS AMOREUXI PESTICIDAL BIOPEPTIDES
Docket No.:	035718/260673
Customer No.:	29122

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APPEAL BRIEF TRANSMITTAL
(PATENT APPLICATION – 37 C.F.R. § 41.37)

1. Transmitted herewith is the APPEAL BRIEF in this application, with respect to the Notice of Appeal filed on September 26, 2006.

2. ☐ Applicant claims small entity status.

3. Pursuant to 37 C.F.R. § 41.20(b)(2), the fee for filing the Appeal Brief is:
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APPEAL BRIEF UNDER 37 CFR § 41.37

This Appeal Brief is filed pursuant to the "Notice of Appeal to the Board of Patent Appeals and Interferences" filed September 26, 2006.

1. ***Real Party in Interest***

The real party in interest in this appeal is E.I. duPont de Nemours and Company, the assignee of the above-referenced patent application.

2. ***Related Appeals and Interferences***

There are no related appeals and/or interferences involving this application or its subject matter.

3. ***Status of Claims***

Claims 1-7, 13-19, 21-31, 38, 40, 42, and 43 were rejected. Claims 11, 12, 20, 39, and 41 were objected to as being dependent upon a rejected base claim, but would be allowable if rewritten in independent form. Claims 28 and 43 were objected to as being dependent upon a

rejected base claim, but would be allowable if rewritten to overcome the rejections under 35 U.S.C. § 112, second paragraph and in independent form. In the Advisory Action dated August 15, 2006, the Examiner noted that Applicants reply to the final Office Action had overcome the rejections under 35 U.S.C. § 112, second paragraph. Claims 8-10, 27 and 32-37 have been canceled. Applicants note that rejected claim 29 depends from claim 28. Accordingly, as claim 28 would be allowable if rewritten in independent form, claim 29 should also be allowable if claim 28 is rewritten in independent form. Claims 1-7, 13-19, 21-26, 30, 31, 38, 40, and 42 are the subject of this appeal.

4. ***Status of Amendments***

No new amendments have been made to the claims.

5. ***Summary of Claimed Subject Matter***

Independent claim 1 of the present application is drawn to an isolated nucleic acid molecule encoding a polypeptide having at least 95% sequence identity to the amino acid sequence set forth in SEQ ID NO:20, wherein said polypeptide retains pesticidal activity against insect pests of the Homopteran or Lepidopteran orders (see, *e.g.*, page 7, lines 3-6 and 12-18; page 8, line 29 through page 9, line 6; page 10, lines 11-16; and page 17, lines 7-20). The claim is also drawn to an isolated nucleic acid molecule comprising a nucleotide sequence having at least 95% sequence identity to the coding sequence set forth in nucleotides 73-249 of SEQ ID NO:17 or nucleotides 64-240 of SEQ ID NO:14, wherein said nucleotide sequence encodes a polypeptide having pesticidal activity against insect pests of the Homopteran or Lepidopteran orders (see, *e.g.*, the pages and line numbers cited above as well as page 10, lines 17-25; and page 16, line 21 through page 17, line 6).

Independent claim 13 of the present application is drawn to a transformed plant comprising in its genome at least one stably incorporated expression cassette comprising a nucleotide sequence operably linked to a promoter that drives expression in a plant cell, wherein said nucleotide sequence encodes a polypeptide having at least 95% sequence identity to the amino acid sequence set forth in SEQ ID NO:20, wherein said polypeptide retains pesticidal

activity against insect pests of the Homopteran or Lepidopteran orders (see, *e.g.*, page 7, lines 3-6 and 12-18; page 8, line 29 through page 9, line 6; page 10, lines 11-16; page 12, lines 19-29; and page 17, lines 7-20). The claim is also drawn to a transformed plant comprising in its genome at least one stably incorporated expression cassette comprising a nucleotide sequence operably linked to a promoter that drives expression in a plant cell, wherein said nucleotide sequence comprises a nucleotide sequence having at least 95% sequence identity to the coding sequence set forth in nucleotides 73-249 of SEQ ID NO:17 or nucleotides 64-240 of SEQ ID NO:14, wherein said nucleotide sequence encodes a polypeptide having pesticidal activity against insect pests of the Homopteran or Lepidopteran orders (see, *e.g.*, the pages and line numbers cited above as well as page 10, lines 17-25; and page 16, line 21 through page 17, line 6).

Independent claim 23 of the present application is drawn to a method for altering plant insect pest resistance comprising stably transforming into a plant cell a nucleotide sequence operably linked to a promoter that drives expression in said plant cell, and regenerating a plant from said plant cell, wherein said nucleotide sequence comprises a nucleotide sequence encoding a polypeptide having at least 95% sequence identity to the amino acid sequence set forth in SEQ ID NO:20, wherein said polypeptide retains pesticidal activity against insect pests of the Homopteran or Lepidopteran orders (see, *e.g.*, page 3, lines 15-27; page 7, lines 3-6 and 12-18; page 8, line 29 through page 9, line 6; page 10, lines 11-16; and page 17, lines 7-20). The claims are also drawn to a method for altering plant insect pest resistance comprising stably transforming into a plant cell a nucleotide sequence operably linked to a promoter that drives expression in said plant cell, and regenerating a plant from said plant cell, wherein said nucleotide sequence comprises a nucleotide sequence having at least 95% sequence identity to the coding sequence set forth in nucleotides 73-249 of SEQ ID NO:17 or nucleotides 64-240 of SEQ ID NO:14, wherein said nucleotide sequence encodes a polypeptide having pesticidal activity against insect pests of the Homopteran or Lepidopteran orders (see, *e.g.*, the pages and line numbers cited above as well as page 10, lines 17-25; and page 16, line 21 through page 17, line 6).

6. ***Grounds of Rejection to Be Reviewed on Appeal***

Issue 1—Whether claims 1-7, 13-19, 21-26, 30, 31, 38, 40, and 42 meet the written description requirement of 35 U.S.C. § 112, first paragraph.

Issue 2—Whether claims 1-7, 13-19, 21-26, 30, 31, 38, 40, and 42 meet the enablement requirement of 35 U.S.C. § 112, first paragraph.

7. ***Grouping of Claims***

The claims stand or fall together. The rejected claims are all "sequence identity claims" which contain limitations that require the isolated nucleic acid molecule of the claims to (i) encode a polypeptide having a specified percent sequence identity to the amino acid sequence set forth in SEQ ID NO:20, or (ii) share a specified percent sequence identity to the coding sequence set forth in nucleotides 73-249 of SEQ ID NO:17 or nucleotides 64-240 of SEQ ID NO:14.

8. ***Argument***

(a) Issue 1—Whether claims 1-7, 13-19, 21-26, 30, 31, 38, 40, and 42 meet the written description requirement of 35 U.S.C. § 112, first paragraph.

Claims 1-7, 13-19, 21-27, 29-31, 38, 40, and 42 were rejected under 35 U.S.C. §112, first paragraph, as failing to comply with the written description requirement. This rejection is respectfully traversed. For the reasons set forth below, the rejection of claims 1-7, 13-19, 21-26, 30, 31, 38, 40, and 42 under 35 U.S.C. §112, first paragraph, as failing to comply with the written description requirement, should be withdrawn.

I. THE CLAIMED INVENTION MEETS THE REQUIREMENTS OF 35 U.S.C. § 112, FIRST PARAGRAPH, FOR WRITTEN DESCRIPTION

An adequate written description for genetic material requires a precise definition, "such as by structure, formula, chemical name, or physical properties." *See*, *Enzo Biochem Inc. v. Gen-Probe, Inc.*, 323 F.3d 956, 962-963, 970 (Fed. Cir. 2002). The goal of the written description requirement is to clearly convey that an applicant has invented the subject matter which is claimed. *See*, for example, *In re Barker*, 559 F.2d 588, 592, (CCPA 1977). To satisfy the

written description requirement, a patent specification must describe the claimed invention in sufficient detail that one skilled in the art can reasonable conclude that the inventor had possession of the claimed invention. *See*, *Moba, B.V. v. Diamond Automation, Inc.* 325 F.3d 1306, 1319, (Fed. Cir. 2003); and *Vas-Cath, Inc. v. Mahurkar*, 935 F2d 1555, 1563 (Fed. Cir. 1991). The written description inquiry focuses on whether the specification reasonably conveys to one skilled in the art whether the Applicants invented the claimed subject matter. Thus, the relevant inquiries are: What is the Applicants' claimed invention? What is now claimed?

A. The Claimed Sequences Are Adequately Described in the Specification

The claimed invention is directed to isolated nucleic acid molecules having specific structural and biological properties. Specifically, nucleotide sequences encoding a polypeptide having at least 95% sequence identity to SEQ ID NO:20, or nucleotide sequences having at least 95% sequence identity to the coding sequence set forth in nucleotides 73-249 of SEQ ID NO:17 or nucleotides 64-240 of SEQ ID NO:14, where the corresponding polypeptides exhibit pesticidal activity against Homopteran or Lepidopteran pests. The specification provides both DNA (*i.e.*, SEQ ID NOs:14 and 17) and amino acid (*i.e.*, SEQ ID NO:20) sequences of a representative embodiment of the claimed sequences. Indeed, the Examiner has acknowledged that the claims drawn to specific sequences would be allowable if rewritten as independent claims. The specification also provides detailed guidance concerning variants of the disclosed sequences, methods for making the same and determining percent identity (see, *e.g.*, page 16, line 21, continuing through page 17, line 20; page 18, lines 6-18; and page 23, line 14, continuing through page 28, line 18). Additionally, as seen in Example 17 (pages 69-70), the specification provides ample guidance for determining the pesticidal activity of the disclosed peptides against Homopteran (*e.g.*, *Perigrinus maidis*, *Myzus persicae* and *Aphis fabeae*) and Lepidopteran (*e.g.*, *Ostrinia nubilalis*) pests. Thus, the nucleotide sequences that fall within the scope of the claims can readily be identified by the methods set forth in the specification.

Applicants note that the description of a representative number of species does not require the description to be of such specificity that it would provide individual support for each species that the genus embraces. *See*, Guidelines for Examination of Patent Applications Under

the 35 U.S.C. 112, First Paragraph, "Written Description" Requirement, 66 Fed. Reg. 1099, 1106 (2001) (referred to herein as the "Guidelines"). Satisfactory disclosure of a "representative number" depends on whether one of skill in the art would recognize that the applicant was in possession of the necessary common attributes or features of the elements possessed by the members of the genus in view of the species disclosed. 66 Fed. Reg. 1099, 1106 (2001). Applicants submit that the knowledge and level of skill in the art would allow a person of ordinary skill to envision the claimed invention, that is, nucleotide sequences encoding a polypeptide having at least 95% sequence identity to SEQ ID NO:20, or nucleotide sequences having at least 95% sequence identity to the coding sequence set forth in nucleotides 73-249 of SEQ ID NO:17 or nucleotides 64-240 of SEQ ID NO:14.

The description of a claimed genus can be by structure, formula, chemical name, or physical properties. *See, Ex parte Maizel*, 27 USPQ2d 1662, 1669 (BPAI 1992), citing *Amgen v. Chugai*, 927 F.2d 1200, 1206 (Fed. Cir. 1991). A genus of DNAs may therefore be described by means of a recitation of a representative number of DNAs defined by nucleotide sequence and falling within the scope of the genus, *or* by means of a recitation of structural features common to the genus, which features constitute a substantial portion of the genus. *See, Regents of the University of California v. Eli Lilly & Co.*, 119 F.3d 1559, 1569 (Fed. Cir. 1997) (referred to herein as "*Lilly*"); *see also*, 66 Fed. Reg. 1099, 1106 (2001). All of the pending claims recite a functional limitation and also require a predictable structure of at least 95% sequence identity to the recited sequences. Under both *Lilly* and the Guidelines, these requirements for function in combination with the recitation of a predictable structure should be sufficient to satisfy the written description requirement.

Applicants note that the Federal Circuit has explicitly stated that

> *Eli Lilly* did not hold that all functional descriptions of genetic material necessarily fail as a matter of law to meet the written description requirement; rather, the requirement may be satisfied if in the knowledge of the art the disclosed function is sufficiently correlated to a particular, known structure.

Amgen, Inc. v. Hoechst Marion Roussel, Inc., 314 F.3d 1313, 1332 (Fed. Cir. 2003). *See also, Moba, B.V. v. Diamond Automation, Inc.*, 325 F.3d 1306, 1320 (noting that "[i]n more recent

cases, however, this court has distinguished *Lilly*" and further noting that in *Enzo Biochem, Inc. v. Gen-Probe, Inc.*, 323 F.3d 956 (Fed. Cir. 2002), "neither the specification nor the deposited biological material recited the precise 'structure, formula, chemical name, or physical properties' required by *Lilly*.").

Example 14 of the "Synopsis of Application of Written Description Guidelines" (available on the Office's website at: http://www.uspto.gov/web/menu/written.pdf) is directed to a generic claim: a protein having at least 95% sequence identity to the sequence of SEQ ID NO:3, wherein the sequence catalyzes the reaction A → B. The synopsis concludes that the generic claim of Example 14 is sufficiently described under § 112, first paragraph, because the single sequence disclosed in SEQ ID NO:3 is representative of the genus, the claim recites a limitation requiring the variants to perform a specific catalytic activity, and the specification provides an assay for identifying those variants capable of performing the specified catalytic activity. The synopsis materials conclude that "one of skill in art would conclude that Applicant was in possession of the necessary common attributes possessed by the members of the genus."

Following the analysis of Example 14, Applicants submit that the present claims satisfy the written description requirements of § 112, first paragraph. Specifically, the claims of the present invention encompass nucleotide sequences encoding a polypeptide having at least 95% sequence identity to SEQ ID NO:20, or nucleotide sequences having at least 95% sequence identity to the coding sequence set forth in nucleotides 73-249 of SEQ ID NO:17 or nucleotides 64-240 of SEQ ID NO:14, where the corresponding polypeptides exhibit pesticidal activity against Homopteran or Lepidopteran pests. As in Example 14, the specification discloses the amino acid sequence of SEQ ID NO:20 and the nucleic acid sequences of SEQ ID NOs:14 and 17, and the claims require the polypeptides to have a specific function (*i.e.*, pesticidal activity). Consequently, the sequences encompassed by the claims are defined by relevant identifying physical and chemical properties. In fact, the common attributes or features of the elements possessed by the members of the genus are that they encode polypeptides having at least 95% sequence identity to SEQ ID NO:20 or are nucleotide sequences having at least 95% sequence identity to the coding sequence set forth in nucleotides 73-249 of SEQ ID NO:17 or nucleotides 64-240 of SEQ ID NO:14, where the encoded polypeptides have pesticidal activity against

Homopteran or Lepidopteran pests. The necessary common features of the claimed genus are clear.

In summary, the description of a representative number of species *does not* require the description to be of such specificity that it would provide individual support for each species that the genus embraces. Rather, "it is sufficient if the originally-filed disclosure would have conveyed to one having ordinary skill in the art that an appellant had possession of the concept of what is claimed." *Ex parte Parks*, 30 USPQ2d 1234, 1236 (BPAI 1992) (citing *In re Anderson*, 471 F.2d 1237, 176 USPQ 331 (CCPA 1973)). Applicants submit that the relevant identifying physical and chemical properties of the disclosed genus would be clearly recognized by one of skill in the art and, consequently, Applicants were in possession of the necessary common attributes or features of the elements possessed by the members of the genus. Accordingly, the rejection of claims 1-7, 13-19, 21-26, 30, 31, 38, 40, and 42 under 35 U.S.C. §112, first paragraph, for lack of written description should be withdrawn.

(b) Issue 2—Whether claims 1-7, 13-19, 21-26, 30, 31, 38, 40, and 42 meet the enablement requirement of 35 U.S.C. § 112, first paragraph.

The Examiner has rejected claims 1-7, 13-19, 21-27, 29-31, 38, 40, and 42 under 35 U.S.C. § 112, first paragraph, and indicated that the specification, while being enabling for nucleic acids encoding SEQ ID NO:20, expression cassettes, host cells, viruses, plants, and seeds comprising the nucleic acids, and methods of using them to alter plant pest resistance, does not reasonably provide enablement for nucleic acids encoding pesticidal proteins with at least 95% sequence identity to SEQ ID NO:20, or pesticide-encoding nucleic acids with at least 95% sequence identity to bases 73-249 of SEQ ID NO:17 or bases 64-240 of SEQ ID NO:14. For the following reasons, the Examiner's reasoning is not well founded and ignores the guidance provided in the specification and in the art, and the rejection of claims 1-7, 13-19, 21-26, 30, 31, 38, 40, and 42 should be reversed.

II. THE CLAIMED INVENTION MEETS THE REQUIREMENTS OF 35 U.S.C. § 112, FIRST PARAGRAPH, FOR ENABLEMENT

To satisfy the requirements of 35 U.S.C. § 112, first paragraph, the specification must teach those skilled in the art to make and use the full scope of the claimed invention without undue experimentation. *Enzo Biochem, Inc. v. Calgene, Inc.*, 188 F.3d 1362, 1371, 52 USPQ2d 1129, 1135 (Fed. Cir. 1999); *Genentech, Inc. v. Novo Nordisk, A/S*, 108 F.3d 1361, 1365, 42 USPQ2d 1001, 1004 (Fed. Cir. 1997); *PPG Inds., Inc. v. Guardian Inds. Corp.*, 75 F.3d 1558, 1564, 37 USPQ2d 1618, 1623 (Fed. Cir. 1996); *In re Wright*, 999 F.2d 1557, 1561-62, 27 USPQ2d 1510, 1513 (Fed. Cir. 1993); *In re Vaeck*, 947 F.2d 488, 495-96, 20 USPQ2d 1438, 1444-45 (Fed. Cir. 1991). "That some experimentation may be required is not fatal, the issue is whether the amount of experimentation required is 'undue.'" *In re Vaeck*, 947 F.2d at 495, 20 USPQ2d at 1444. The enablement section of 35 U.S.C. § 112, first paragraph, "requires that the scope of the claims must bear a reasonable correlation to the scope of enablement provided by the specification to persons of ordinary skill in the art." *In re Fisher*, 427 F.2d 833, 839, 166 USPQ 18, 24 (CCPA 1970). In order to determine whether the present claims are enabled, an analysis of the teachings of the specification must be performed as well as an inquiry into the knowledge of persons of ordinary skill in the art. *In re Bowen*, 492 F.2d 859, 861, 181 USPQ 48, 50 (CCPA 1974).

A. The Claimed Invention Is Fully Taught in the Specification.

The claims are generally drawn to nucleotide sequences encoding a polypeptide having at least 95% sequence identity to SEQ ID NO:20, or nucleotide sequences having at least 95% sequence identity to the coding sequence set forth in nucleotides 73-249 of SEQ ID NO:17 or nucleotides 64-240 of SEQ ID NO:14, and in addition require that the nucleotide sequences encode a polypeptide that is pesticidal for Homopteran or Lepidopteran pests (*e.g.*, the polypeptide set forth in SEQ ID NO:20). The specification teaches those skilled in the art how to make the claimed nucleotide sequences and provides examples of such sequences. As described above, the specification also provides detailed guidance concerning variants of the disclosed sequences, methods for making the same and determining percent identity (see, *e.g.*, page 16,

line 21, continuing through page 17, line 20; page 18, lines 6-18; and page 23, line 14, continuing through page 28, line 18), and methods for determining the pesticidal activity of the disclosed peptides against Homopteran (*e.g.*, *Perigrinus maidis*, *Myzus persicae* and *Aphis fabeae*) and Lepidopteran (*e.g.*, *Ostrinia nubilalis*) pests (see, *e.g.*, working Example 17 on pages 69-70). Accordingly, the skilled artisan could choose among possible modifications to produce polypeptides within the parameters set forth in the claims and then test these modified variants to determine if they retain pesticidal activity.

B. One of Skill in the Art Can Make and Use the Invention Based on the Teachings of the Specification

The Federal Circuit has repeatedly stated that enablement is not precluded by the necessity for some experimentation, so long as the experimentation needed to practice the invention is not undue. *In re Wands* 8 USPQ2d 1400 (Fed Cir 1988). Furthermore, a considerable amount of experimentation is permissible, if it is merely routine, or if the specification provides a reasonable amount of guidance in which the experimentation should proceed. *Id.* In the instant case, the quantity of experimentation required to practice independent claims 1, 13 and 23 amounts to two steps. First, generating a nucleotide sequence encoding a polypeptide having at least 95% sequence identity to the amino acid sequence set forth in SEQ ID NO:20, or a nucleotide sequence having at least 95% sequence identity to the coding sequence set forth in nucleotides 73-249 of SEQ ID NO:17 or nucleotides 64-240 of SEQ ID NO:14. Second, assaying the encoded polypeptide for functional activity. Such assays, while known in the art, have further been presented in the specification. See, for example, working Examples 5 (pages 56-57), 6 (page 58) and 17 (pages 69-70).

One of skill in the art would appreciate that both of these steps are within the skill of those in the art and that this degree of experimentation is not considered undue. "[A] specification disclosure which contains a teaching of the manner and process of making and using the invention in terms which correspond in scope to those used in describing and defining the subject matter sought to be patented *must* be taken as in compliance with the enabling requirement of the first paragraph of § 112 *unless* there is reason to doubt the objective truth of

the statements contained therein which must be relied on for enabling support." *In re Marzocchi*, 439 F.2d 220, 223, 169 USPQ 367, 369 (CCPA 1971) (emphasis in original).

Applicants note that it was customary in the art at the time of the invention to make and assay a number of sequences for a desired function in order to achieve the best results. For example, common techniques involve what is often referred to as "shuffling," as described for example in U.S. Patent No. 5,837,458, issued November 17, 1998. With such techniques, it is common to mutagenize individual sequences or a set of sequences which are then assayed for a desired activity. Such techniques may even make use of a library of sequences which is recursively mutagenized, screened for function using a functional assay, and re-mutagenized in order to find a sequence exhibiting optimal function. Examples of the use of such techniques include: Minshull and Stemmer (*Curr. Opin. Chem. Bio.* 3:284-290 (1999)) and Christians *et al.* (*Nature Biotech.* 17: 259-264 (1999)). Such experiments are designed and are intended to encompass the generation and testing of a number of variant sequences for a desired function. As indicated by these and other publications in the art, this level of experimentation was considered routine in the art and thus would not be considered "undue experimentation" under *Wands*.

Additionally, at the time of the invention, it was routine to mutate amino acids in a polypeptide and then test the altered polypeptide for activity. This is proven by the references cited by the Examiner in the Office Action (*i.e.*, Lazar *et al.*, *Molecular & Cellular Biology* 8:1247-1252 (1988) and Hill *et al.*, *Biochemical & Biophysical Research Communications* 244:573-577 (1998)). The Lazar reference (Evidence Appendix A) and the Hill reference (Evidence Appendix B) both demonstrate that one of skill in the art well before the 2002 priority date of the present application could make substitutions in polypeptide sequences and test for activity. Nothing more is required in the present application.

As assays for determining whether the modified sequences would retain activity were disclosed, one of skill in the art as of the filing date of the present application would have been able to make such modifications and test them for pesticidal activity. Nothing more is required to fully enable the claims. Accordingly, one of skill in the art would be able to determine the

functionality of polypeptides encompassed by the claimed invention without resorting to undue experimentation and therefore the enablement requirement is satisfied.

C. The Examiner's Reasoning Is Not Well-Founded

Applicants have provided the exemplary nucleotide sequences of SEQ ID NOs:14 and 17 and the exemplary amino acid sequence of SEQ ID NO:20. The claimed nucleotide sequences of the invention vary from the exemplary sequences by structural parameters (*i.e.*, at least 95% sequence identity to the coding sequence set forth in nucleotides 73-249 of SEQ ID NO:17 or nucleotides 64-240 of SEQ ID NO:14). Guidance for determining percent identity of sequences is provided in the specification on page 23, line 14, continuing through page 28, line 18.

Moreover, independent claims 1, 13 and 23, in addition to requiring a structural component, specify that the nucleotide sequence encodes a polypeptide which is pesticidal for pests of the Homopteran or Lepidopteran orders, and therefore these claims (and claims dependent thereon) encompass functional variants. Guidance regarding alterations that allow the sequence to retain the specified pesticidal activity is also provided. See, for example, page 18, lines 6-18 of the specification, which lists a number of exemplary references for such procedures. In addition, methods for assaying pesticidal activity of proteins are routine in the art and are also described in the specification, for example in the experimental section in working examples such as Example 5 (pages 56-57), Example 6 (page 58) and Example 17 (pages 69-70).

On page 10, lines 5-10 of the Office Action, the Examiner reasons that to enable the claims, one must make and test all possible combinations of nucleic acids falling within the scope of the claim.

> Making all possible single amino acid substitutions, in an 58 amino acid long protein like that of SEQ ID NO:20 would require making and analyzing 19^{58} nucleic acids; these proteins would have 98.3% identity to SEQ ID NO:20. Because nucleic acids encoding proteins with 90% identity to SEQ ID NO:20 would encode proteins with 2 amino acid substitutions, many more than 19^{58} nucleic acids would need to be made and analyzed.

The Examiner's analysis is improper. As held by the court in *In re Borkowski*, 422 F.2d 904, 909, 164 USPQ 642, 645 (CCPA 1970), it is inappropriate "to study appellants' disclosure, to formulate a conclusion as to what he (the examiner) regards as the broadest invention supported by the disclosure, and then to determine whether appellants' claims are broader than the examiner's conception of what 'the invention' is." In the present case, the methods and examples disclosed in the specification readily teach one of skill in the art to make and test nucleotide sequences encoding a polypeptide having at least 95% sequence identity to SEQ ID NO:20, or nucleotide sequences having at least 95% sequence identity to the coding sequence set forth in nucleotides 73-249 of SEQ ID NO:17 or nucleotides 64-240 of SEQ ID NO:14.

Under the facts of the present application, one skilled in the art would understand whether a particular protein has at least 95% sequence identity with SEQ ID NO:20 or whether a particular nucleotide sequence has at least 95% sequence identity to the coding sequence set forth in nucleotides 73-249 of SEQ ID NO:17 or nucleotides 64-240 of SEQ ID NO:14 as set forth in the claims. In addition, functional assays are disclosed in the specification that provide sufficient guidance for one skilled in the art to determine whether a particular polynucleotide is within the scope of the claims. Thus, the claims are fully enabled.

D. That Some Experimentation May Be Necessary Does Not Indicate That the Claims Are Not Enabled

It is recognized that in unpredictable art areas, the court has refused to find broad generic claims enabled where the corresponding specifications only demonstrate the enablement of one or very few embodiments and do not demonstrate with reasonable specificity how to make and use other potential embodiments across the full scope of the claim. *See*, for example, *In re Goodman*, 11 F.3d 1046, 1050-52, 29 USPQ2d 2010, 2013-15 (Fed. Cir. 1993); *Amgen, Inc. v. Chugai Pharm. Co.*, 927 F.2d 1200, 1212-14, 18 USPQ2d 1016, 1026-28 (Fed. Cir. 1991); *In re Vaeck*, 947 F.2d at 496, 20 USPQ2d at 1445. The court has explained that enablement is lacking in those cases because the undescribed embodiment cannot be made based on the disclosure in the specification, without undue experimentation. However, the court has made clear that the question of undue experimentation is a matter of degree. The fact that some experimentation is

necessary does not preclude enablement; what is required is that the amount of experimentation "must not be unduly extensive." *Atlas Powder Co. v. E.I. DuPont de Nemours & Co.*, 750 F.2d 1569, 1576, 224 USPQ 409, 413 (Fed. Cir. 1984). The Patent and Trademark Office Board of Appeal has indicated: "the test is not merely quantitative, since a considerable amount of experimentation is permissible, if it is merely routine, or if the specification in question provides a reasonable amount of guidance with respect to the direction in which the experimentation should proceed to enable the determination of how to practice a desired embodiment of the invention claimed." *Ex Parte Jackson*, 217 USPQ 804, 807 (BPAI 1982).

In the present case, all the Examiner has established is that some experimentation would be required to make and use other embodiments of the claimed invention. What the Examiner has not done is perform the fact-finding needed in order to reach a proper conclusion of undue experimentation. The Examiner has not relied upon any evidence in support of this rejection which would establish that making and testing other sequences beyond those described in the present specification amounts to undue experimentation. In fact, the Examiner has ignored the guidance in the specification and the teachings of the prior art. The references cited by the Examiner support the position that the procedures described in the specification for making and testing modified sequences are routine in the art. The Examiner makes the rejection based upon unsupported opinions.

E. The Examiner Mischaracterizes the Cited References

The Examiner argues that making substitutions is not predictable and cites Lazar *et al.*, *Molecular & Cellular Biology* 8:1247-1252 (1988), in support of this position. The Examiner indicates that the "conservative" substitution of glutamic acid for aspartic acid at position 47 reduced biological function of transforming growth factor alpha (TGF-α) while "nonconservative" substitutions with alanine or asparagine had no effect (Office Action, page 9, lines 14-17). The Examiner fails to consider the entire teachings of the reference.

First, the Lazar reference is drawn to studying TGF-α. TGF-α is a mammalian polypeptide and is in no way related to the *Androctonus amoreuxi* proteins of the present invention. The reference relating to TGF-α does not bear any relevance to the recited

Androctonus amoreuxi sequences. Secondly, with respect to the modifications described by Lazar *et al.*, two amino acids of TGF-α which were known to be conserved among the family of EGF-like polypeptides were modified. It would come as little surprise to one skilled in the art that the modification of such a conserved amino acid should lead to the loss of function described by the authors.

The Examiner additionally cites Hill *et al.*, *Biochemical & Biophysical Research Communications* 244:573-577 (1998), as supporting the position that substitution of a residue with a conservative amino acid can drastically reduce enzyme activity. The Examiner cites Hill *et al.* as teaching that when three histidines that are maintained in ADP-glucose pyrophosphorylase across several species are substituted with the "nonconservative" amino acid glutamine, there is little effect on enzyme activity, while the substitution of one of those histidines with the "conservative" amino acid arginine drastically reduced enzyme activity (Office Action, page 9, line 17, continuing through page 10, line 1).

First, the Hill reference is drawn to studying ADP-glucose pyrophosphorylase. The polypeptide is in no way related to the *Androctonus amoreuxi* proteins of the present invention. The reference relating to ADP-glucose pyrophosphorylase does not bear any relevance to the claimed *Androctonus amoreuxi* sequences. Secondly, with respect to the modifications described by Hill *et al.*, the modified residues were conserved among bacterial and plant ADP-glucose pyrophosphorylases. As set forth in the first line of the abstract, "[t]wo **absolutely conserved** histidines and a third **highly conserved** histidine are noted in 11 bacterial and plant ADP-glucose pyrophosphorylases" (emphasis added). These **absolutely** and **highly conserved** histidines were mutagenized and characterized in the paper. It would come as little surprise to one skilled in the art that the modification of one of these conserved amino acids should lead to the loss of function described by the authors.

In summary, in establishing nonenablement, the burden rests initially with the Examiner to substantiate the unpredictability of the art and that, given the unpredictability, the specification does not provide sufficient information to guide those of skill to make and use the claimed invention across the full scope of the claims. In the present case, a clear goal is disclosed. Furthermore, guidance is provided for making the claimed sequences and assays are provided to

determine whether modified sequences would encode proteins that retain activity. Consequently, contrary to the conclusions stated in the Office Action, the quantity of experimentation necessary and the amount of guidance presented in the specification is sufficient to enable the claims. Accordingly, the rejection of claims 1-7, 13-19, 21-26, 30, 31, 38, 40, and 42 under 35 U.S.C. §112, first paragraph, for lack of enablement should be withdrawn.

9. ***Claims Appendix***

An appendix containing a copy of the claims involved in the appeal.

10. ***Evidence Appendix***

An appendix containing copies of the evidence submitted as follows:

Evidence Appendix A – Lazar *et al.* (1988)

Evidence Appendix B – Hill *et al.* (1998)

CONCLUSION

Appellants maintain that the Examiner has failed to carry her burden of establishing that the claims are not patentable because she has (a) failed to prove that the application does not adequately describe the claimed invention and (b) failed to establish that it would require undue experimentation to practice the claimed invention. Accordingly, claims 1-7, 13-19, 21-26, 30, 31, 38, 40, and 42 are allowable. For these reasons, presented in detail above, Appellants respectfully request that the rejections be reversed.

It is not believed that extensions of time are required. However, in the event that extensions of time are necessary to allow consideration of this paper, such extensions are hereby petitioned under 37 CFR § 1.136(a), and any fee required therefore is hereby authorized to be charged to Deposit Account No. 16-0605.

Respectfully submitted,

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Nora C. Martinez

CLAIMS APPENDIX

APPEALED CLAIMS

1. (Previously Presented) An isolated nucleic acid molecule having a nucleotide sequence selected from the group consisting of:

(a) a nucleotide sequence encoding a polypeptide comprising the amino acid sequence set forth in SEQ ID NO:20;

(b) a nucleotide sequence comprising the coding sequence set forth in nucleotides 73-249 of SEQ ID NO:17 or nucleotides 64-240 of SEQ ID NO:14;

(c) a nucleotide sequence encoding a polypeptide having at least 95% sequence identity to the amino acid sequence set forth in SEQ ID NO:20, wherein said polypeptide retains pesticidal activity against insect pests of the Homopteran or Lepidopteran orders; and

(d) a nucleotide sequence having at least 95% sequence identity to the coding sequence set forth in nucleotides 73-249 of SEQ ID NO:17 or nucleotides 64-240 of SEQ ID NO:14, wherein said nucleotide sequence encodes a polypeptide having pesticidal activity against insect pests of the Homopteran or Lepidopteran orders.

2. (Original) An expression cassette comprising a nucleic acid molecule of claim 1, wherein said nucleotide sequence is operably linked to a promoter.

3. (Original) The expression cassette of claim 2, wherein said promoter is selected from the group consisting of constitutive, inducible, and tissue-preferred promoters.

4. (Original) The expression cassette of claim 2, wherein said promoter is a vascular tissue-preferred promoter.

5. (Original) A host cell expressing a polypeptide encoded by any one of the nucleic acid molecules of claim 1.

6. (Original) The host cell of claim 5, wherein the host cell is selected from the group consisting of fungi, yeast, plant, mammal, and insect cells.

7. (Previously Presented) A virus comprising the isolated nucleic acid of claim 1.

13. (Previously Presented) A transformed plant comprising in its genome at least one stably incorporated expression cassette comprising a nucleotide sequence operably linked to a promoter that drives expression in a plant cell, wherein said nucleotide sequence is selected from the group consisting of:

(a) a nucleotide sequence encoding a polypeptide comprising the amino acid sequence set forth in SEQ ID NO:20;

(b) a nucleotide sequence comprising the coding sequence set forth in nucleotides 73-249 of SEQ ID NO:17 or nucleotides 64-240 of SEQ ID NO:14;

(c) a nucleotide sequence encoding a polypeptide having at least 95% sequence identity to the amino acid sequence set forth in SEQ ID NO:20, wherein said polypeptide retains pesticidal activity against insect pests of the Homopteran or Lepidopteran orders; and

(d) a nucleotide sequence having at least 95% sequence identity to the coding sequence set forth in nucleotides 73-249 of SEQ ID NO:17 or nucleotides 64-240 of SEQ ID NO:14, wherein said nucleotide sequence encodes a polypeptide having pesticidal activity against insect pests of the Homopteran or Lepidopteran orders.

14. (Original) The transformed plant of claim 13, wherein said promoter is selected from the group consisting of constitutive, inducible, and tissue-preferred promoters.

15. (Original) The transformed plant of claim 13, wherein said promoter is a vascular tissue-preferred promoter.

16. (Original) The transformed plant of claim 13, wherein said promoter is an insect-inducible promoter.

17. (Original) The transformed plant of claim 13, wherein said plant is a crop plant selected from the group consisting of maize, wheat, sorghum, rice, barley, soybean, alfalfa, sunflower, *Brassica*, and tomato.

18. (Original) The transformed plant of claim 17, wherein said crop plant is rice.

19. (Previously Presented) A transformed seed of the plant of claim 13, comprising in its genome said at least one stably incorporated expression cassette.

21. (Original) The plant of claim 13, wherein said plant exhibits altered insect resistance.

22. (Previously Presented) The plant of claim 21, wherein said insect resistance is impacting insects selected from the group consisting of Homopteran and Lepidopteran species.

23. (Previously Presented) A method for altering plant insect pest resistance, said method comprising stably transforming into a plant cell a nucleotide sequence operably linked to a promoter that drives expression in said plant cell, and regenerating a plant from said plant cell, wherein said nucleotide sequence comprises a nucleotide sequence selected from the group consisting of:

(a) a nucleotide sequence encoding a polypeptide comprising the amino acid sequence set forth in SEQ ID NO:20;

(b) a nucleotide sequence comprising the coding sequence set forth in nucleotides 73-249 of SEQ ID NO:17 or nucleotides 64-240 of SEQ ID NO:14;

(c) a nucleotide sequence encoding a polypeptide having at least 95% sequence identity to the amino acid sequence set forth in SEQ ID NO:20, wherein said polypeptide retains pesticidal activity against insect pests of the Homopteran or Lepidopteran orders; and

(d) a nucleotide sequence having at least 95% sequence identity to the coding sequence set forth in nucleotides 73-249 of SEQ ID NO:17 or nucleotides 64-240 of SEQ ID

NO:14, wherein said nucleotide sequence encodes a polypeptide having pesticidal activity against insect pests of the Homopteran or Lepidopteran orders.

24. (Original) The method of claim 23, wherein said promoter is selected from the group consisting of constitutive, inducible, and tissue-preferred promoters.

25. (Original) The method of claim 23, wherein said promoter is a vascular tissue-preferred promoter.

26. (Original) The method of claim 23, wherein said promoter is an insect-inducible promoter.

30. (Original) The method of claim 23, wherein said plant is a crop plant selected from the group consisting of maize, wheat, sorghum, rice, barley, soybean, alfalfa, sunflower, *Brassica*, and tomato.

31. (Previously Presented) The method of claim 23, wherein said insect resistance impacts insects selected from the group consisting of Homoptera and Lepidoptera.

38. (Previously Presented) The nucleic acid molecule of claim 1, wherein said nucleotide sequence encoding said polypeptide further comprises an operably linked sequence encoding a signal peptide.

40. (Previously Presented) The transformed plant of claim 13, wherein said nucleotide sequence encoding said polypeptide further comprises an operably linked sequence encoding a signal peptide.

42. (Previously Presented) The method of claim 23, wherein said nucleotide sequence encoding said polypeptide further comprises an operably linked sequence encoding a signal peptide.

EVIDENCE APPENDIX

MOLECULAR AND CELLULAR BIOLOGY, Mar. 1988, p. 1247–1252
0270-7306/88/031247-06$02.00/0

Vol. 8, No. 3

Transforming Growth Factor α: Mutation of Aspartic Acid 47 and Leucine 48 Results in Different Biological Activities

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To study the relationship between the primary structure of transforming growth factor α (TGF-α) and some of its functional properties (competition with epidermal growth factor (EGF) for binding to the EGF receptor and induction of anchorage-independent growth), we introduced single amino acid mutations into the sequence for the fully processed, 50-amino-acid human TGF-α. The wild-type and mutant proteins were expressed in a vector by using a yeast α mating pheromone promoter. Mutations of two amino acids that are conserved in the family of the EGF-like peptides and are located in the carboxy-terminal part of TGF-α resulted in different biological effects. When aspartic acid 47 was mutated to alanine or asparagine, biological activity was retained; in contrast, substitutions of this residue with serine or glutamic acid generated mutants with reduced binding and colony-forming capacities. When leucine 48 was mutated to alanine, a complete loss of binding and colony-forming abilities resulted; mutation of leucine 48 to isoleucine or methionine resulted in very low activities. Our data suggest that these two adjacent conserved amino acids in positions 47 and 48 play different roles in defining the structure and/or biological activity of TGF-α and that the carboxy terminus of TGF-α is involved in interactions with cellular TGF-α receptors. The side chain of leucine 48 appears to be crucial either indirectly in determining the biologically active conformation of TGF-α or directly in the molecular recognition of TGF-α by its receptor.

Transforming growth factor α (TGF-α) is a polypeptide of 50 amino acids. First isolated from a retrovirus-transformed mouse cell line (9), it has subsequently been found in human tumor cells (10, 29), in the early rat embryo (18), and recently in cell cultures from the pituitary gland (23). TGF-α appears to be closely related to epidermal growth factor (EGF) structurally and functionally (19, 20). The two peptides apparently bind to the same receptor, and both induce anchorage-independent growth of certain nontransformed cells, such as NRK cells, in the presence of TGF-β (1).

Comparison of amino acid sequences reveals about 35% homology among the EGF-like peptides (rat [27], mouse [25], and human [13] EGFs and rat [19] and human [12] TGF-αs). Some viral peptides (Shope fibroma growth factor [6], vaccinia growth factor [2], and myxoma growth factor [30]) also share homologies with the EGF-like peptides.

If TGF-α is involved in transformation, a TGF-α antagonist could be an important therapeutic tool in the treatment of certain types of malignancies. An understanding of the conformational and dynamic properties of the TGF-α molecule is basic to the design of an antagonist. A hypothetical antagonist would bind to the same receptor as TGF-α, but would not induce the series of proliferative and transforming events induced by TGF-α. To obtain such a molecule it is necessary to dissociate interactions responsible for binding from those involved in signal transduction. We decided to approach the problem by way of site-directed mutagenesis of a human sequence of TGF-α. In this report we describe our first series of mutations, which were carried out at residues Asp-47 and Leu-48, in the carboxy-terminal part of TGF-α; these two amino acids are highly conserved in the EGF-like family of peptides. We show that these two adjacent residues play different roles in the structure and/or function of TGF-α.

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MATERIALS AND METHODS

Cells. Normal rat kidney (NRK) cells were grown in Dulbecco modified Eagle medium containing 10% (vol/vol) calf serum.

TGF-α gene. The sequence of the 50-amino-acid human TGF-α was originally derived from a human TGF-α precursor cDNA (12). The coding sequence is preceded by an ATG methionine codon and followed by a TAA stop codon and is flanked by *Eco*RI restriction sites. This *Eco*RI fragment combines the 59-base-pair *Eco*RI-*Nco*I fragment from plasmid pTE5 (12) with the 111-base-pair *Nco*I-*Eco*RI fragment from plasmid pyTE2 (11). The resulting *Eco*RI fragment was inserted in M13mp18 for site-directed mutagenesis.

Synthesis and purification of oligonucleotides and oligonucleotide-directed mutagenesis. The synthesis and purification of 20- to 27-nucleotide oligonucleotides were carried out as described previously (31). The one or two nucleotides responsible for the mutation were located in the middle of the oligonucleotide. Mutagenesis was performed by published procedures (21, 33). The sequences of the mutant clones were verified by the method of Sanger et al. (25).

Yeast shuttle vector. The vector YEp70αT contains a yeast α-factor pheromone promoter and prepro sequence for the expression of TGF-α (15). The mutant TGF-α coding sequence was inserted in the *Eco*RI site of plasmid YEp70αT and expressed in the form of a fusion protein consisting of 92 amino acids from the prepro sequence of the yeast α factor attached to the amino terminus of TGF-α (28). The yeast cleaves the precursor and secretes TGF-α with 8 amino acids fused to it (4 are encoded by the prepro sequence of α-factor, and the other 4 are encoded by the DNA sequence added to insert of the TGF-α gene). The last of these residues is a methionine, which allows the cleavage of the secreted fusion

protein by cyanogen bromide (CNBr) and the release of a mature TGF-α (50 amino acids) (see Results).

Yeast strain and transformation. The yeast *Saccharomyces cerevisiae* 20B-12 (*MATα trp1 pep4-3*) (17) was obtained from the Yeast Genetics Stock Center, Berkeley, Calif. *S. cerevisiae* 20B-12 was grown in YEPD medium (1% yeast extract [Difco Laboratories], 2% Bacto-Peptone [Difco], 2% glucose). When the culture reached an optical density at 660 nm of 1, spheroplasts were prepared (14) for transformation. For each transformation we used 10 to 15 μg of purified plasmid DNA.

Partial purification of TGF-α mutants. At 3 days after transformation, five individual colonies of transformants were grown to saturation in YEPD medium. The amount of protein in the yeast medium was measured by the method of Bradford (3), and the amount of mutant TGF-α secreted in the yeast medium was determined by radioimmunoassay. The clones which secrete the highest amount of mutant TGF-α were used to grow a 1-liter culture in YNB-CAA medium (0.67% yeast nitrogen base, 20 g of glucose per liter, 10 g of Casamino Acids [Difco] per liter). After the culture reached saturation (optical density at 660 nm of 10 to 12) (48 h in an air shaker at 30°C), the yeast conditioned medium was dialyzed extensively against 1 M acetic acid in 3,000-molecular-weight cutoff dialysis tubing. Usually 250 ml of dialyzed culture was lyophilized, suspended in 10 ml of 70% formic acid, and treated with CNBr (molar excess of 500) for 20 h at room temperature. The CNBr was subsequently evaporated, and the samples were lyophilized. CNBr-treated samples were suspended in 1 ml of 1 M acetic acid, loaded on a Bio-gel P30 column (30 by 1.5 cm [Bio-Rad Laboratories]), and eluted with 1 M acetic acid. Fractions of 1 ml were collected. Aliquots were lyophilized, suspended in binding buffer (minimum essential medium containing 1 mg of bovine serum albumin per ml and 25 mM HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; pH 7.4]), neutralized if necessary to pH 7.4, and tested in EGF-binding competition and soft-agar assays, as well in radioimmunoassay.

Radioimmunoassays. The amounts of TGF-α secreted in the yeast medium were determined by radioimmunoassay with the immunoglobulin G fraction of a polyclonal antibody, 34D, raised against recombinant human TGF-α (4), in 0.1 M Tris (pH 7.5)–0.15 M NaCl–2.5 mg of bovine serum albumin per ml. The amounts of partially purified TGF-α present in the P30 column fractions were measured by using the Biotope RIA kit with polyclonal antibody against human TGF-α (a gift from W. Hargreaves, Biotope), under denaturing conditions, as recommended by the supplier.

EGF binding competition assay and soft agar assay. Both EGF-binding competition and soft-agar assays have been described previously (1).

RESULTS

Rationale for mutations in the carboxyl terminus of TGF-α. Figure 1 shows the amino acid sequence of TGF-α in which the residues that are conserved among all the EGF-like peptides described thus far (EGF, TGF-α, and EGF-like viral proteins) are enclosed in bold circles. Among the 11 conserved amino acids, there are 6 Cys and 2 Gly residues, which presumably play essential roles in determining the overall conformation of the molecule. We concentrated on the two conserved amino acids in the carboxyl terminus, Asp-47 and Leu-48. The Asp in position 47 is conserved among the EGFs and TGF-α (human or murine), but not among the EGF-like viral proteins (vaccinia growth factor, Shope fibroma growth factor, or myxoma growth factor), whereas Leu 48 is conserved among all the EGF-like peptides so far described. In both mouse and human EGF, the two corresponding residues (Asp-46 and Leu-47) are located near the surface of the protein (8, 22, 22a). We designed a series of mutations in these two positions.

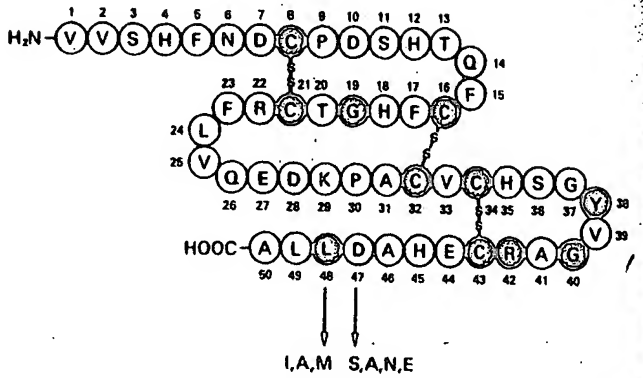

FIG. 1. Mutations in the carboxy terminus of human TGF-α. The amino acids conserved in all the family of EGF-like growth factors (human and murine EGFs and TGFs, as well as the gene products of the vaccinia virus [vaccinia growth factor], the Shope fibroma virus [Shope fibroma growth factor], and the myxoma virus [myxoma growth factor]) are enclosed in bold circles. The mutations of amino acids at positions 47 and 48 are indicated. Symbols: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; Y, Tyr.

Asp-47 has been mutated to Glu, Asn, Ser, and Ala. Glu was chosen because it has the same charge as and a larger size than Asp; Asn has a similar side-chain structure, but is uncharged; Ser is smaller but still polar; Ala is smaller and nonpolar.

Leu 48 has been mutated to Ile and Met, which are both large, nonpolar residues like Leu, and to Ala, which is nonpolar but smaller. We introduced the chosen mutations by site-directed mutagenesis of the cloned human TGF-α gene, using synthetic oligonucleotides.

Construction of the yeast α mating pheromone–human TGF-α plasmid. The TGF-α expression vector pyTE1 (Fig. 2) was constructed by using plasmid YEp70αT (15) which contains the 2μm origin of replication and yeast *TRP1* gene for its replication and selective maintenance, respectively. YEp70αT also contains the yeast α-factor promoter, the α-factor prepro sequence coding for 89 amino acids, and the sequence for 3 amino acids resulting from the introduction of *Xba*I and *Eco*RI sites. The human mature TGF-α sequence (12) is contained in a 170-base-pair *Eco*RI fragment which includes an ATG (Met) codon preceding the sequence of TGF-α and a TAA (stop) codon followed by 8 nucleotides. This TGF-α sequence was inserted in the unique *Eco*RI site of YEp70αT. Clones with the proper orientation were selected, and DNA was isolated for yeast transformation.

Measurement of TGF-α secreted by *S. cerevisiae*. The amount of total proteins secreted into the yeast culture was 10 ± 1 μg/ml for wild-type as well as mutant TGF-α as determined by the method of Bradford (3). Before further purification was attempted, we wanted to determine whether the mutated TGF-α proteins were being secreted by the yeast. The low pH of the yeast medium, as well as the acidic proteins secreted in the yeast culture, precluded biological assay of secreted mutants. Therefore, immunological meth-

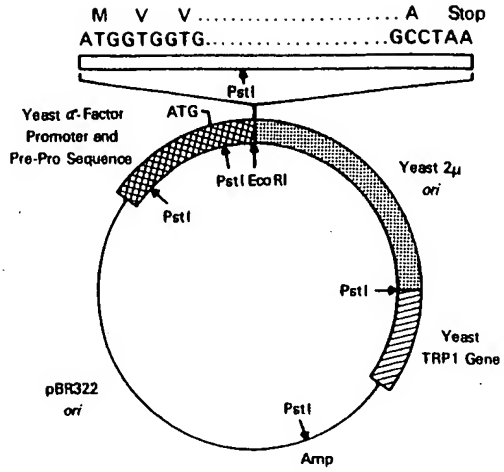

FIG. 2. Structure of the *S. cerevisiae* 8.2-kilobase shuttle vector pyTE1. The secretion of the TGF-α gene is under the transcriptional control of the yeast α-factor promoter and prepro sequence (▩). The yeast 2μm origin of replication (▨) and the selective yeast *TRP1* gene (▧) are indicated. The TGF-α gene, preceded by an initiation (ATG) codon and followed by a stop (TAA) codon, is inserted in the *Eco*RI site. Details are given in Materials and Methods and in Results.

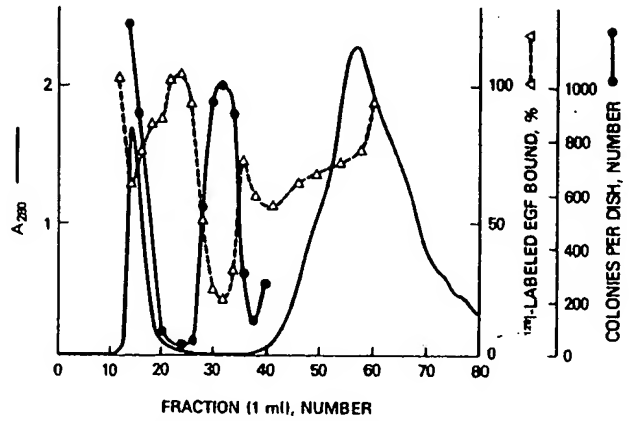

FIG. 3. Purification of yeast-secreted wild-type TGF-α. The purification procedure is described in Materials and Methods and in Results. Aliquots of every other fraction of the Bio-Gel P30 column were tested for their abilities to compete with ^{125}I-EGF for binding to the EGF receptor (Δ) and to induce colony formation (>62 μm) on NRK cells in soft agar in the presence of TGF-β (1 ng/ml) (●). The A_{280} profile of the proteins was determined (——).

ods were used. Wild-type and mutant TGF-α's were secreted at a level of 100 to 200 ng/ml and 10 to 500 ng/ml, respectively (as determined by radioimmunoassay with polyclonal antibody 34D). We thus estimate that the percentage of TGF-α secreted in the yeast culture is at least 1% of the total protein secreted. We cannot yet assess whether the variations in the levels of secretion of different mutant TGF-α proteins are real or whether one single-amino-acid substitution drastically affects the recognition by the antibody. The latter hypothesis is the more likely, since the use of another polyclonal antibody (Biotope) under denaturing conditions enabled us to detect certain TGF-α mutants (such as [Ala 47]–TGF-α, in which the amino acid in position 47 of human TGF-α is mutated to an alanine) that were poorly detected by 34D, under nondenaturing as well as denaturing conditions. After the amount of TGF-α mutant proteins was estimated, the medium was extensively dialyzed against 1 M acetic acid and lyophilized as described in Materials and Methods.

Partial purification of yeast-secreted TGF-α. Although the yeast shuttle vector was constructed in such a way as to secrete TGF-α with 8 amino acids fused to the N terminus, it was often observed that a significant fraction of the secreted TGF-α was in a higher-molecular-weight fragment corresponding to the size expected from an uncleaved (unprocessed) 92-amino-acid fusion protein. Since a Met had been introduced at the N terminus of TGF-α and since TGF-α contains no Met in its sequence, CNBr treatment could be used to cleave either of these 8- or 92-amino-acid N-terminal peptides and release the complete 50-amino-acid TGF-α. Indeed, CNBr treatment of yeast-secreted proteins resulted in the conversion of high-molecular-weight TGF-α into the 6,000-molecular-weight species, as revealed by Western immunoblot (data not shown).

CNBr-cleaved samples (see Materials and Methods) were purified on a Bio-Gel P30 column. Figure 3 shows the elution profile of the proteins, as well as the results of a radioreceptor assay and a soft-agar assay performed on aliquots of the column fractions. The A_{280} profile shows two major peaks of eluted proteins, one corresponding to the void volume and the other one to proteins of molecular weight <3,000. Aliquots of the column fractions were tested for their ability to compete with ^{125}I-EGF for binding to the receptor. The fractions that were the most active in this assay were located between the two major protein peaks, in an area where relatively few proteins eluted. Although some activity was found in the first protein peak (void volume), this was considerably reduced on treatment with stronger CNBr (data not shown).

Aliquots of each fraction were also tested for their ability to induce anchorage-independent growth of NRK cells in soft agar in the presence of TGF-β (1 ng/ml). The receptor binding and colony-forming activity superimposed almost exactly (Fig. 3). Analysis by polyacrylamide gel electrophoresis with silver staining, as well as by Western blot, of the column fractions shows that our purification procedure (CNBr cleavage followed by P30 sizing column) eliminates high-molecular-weight proteins (data not shown). Since pure TGF-α migrates in a broad band on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (32), this technique cannot be used for proper assessment of the degree of separation of TGF-α from low-molecular-weight contaminating proteins. Nevertheless, within our detection levels the amounts of TGF-α present in the column fractions (detected by radioimmunoassay using the antibody from Biotope) correlated with the amounts observed on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (data not shown).

Comparison of binding and colony-forming activity of TGF-α partially purified from yeast media. It was important to show that wild-type TGF-α secreted from *S. cerevisiae* had the expected biological properties and that its activity in soft-agar and radioreceptor assays was equivalent. For these assays, the amount of EGF-competing activity present in the most active fraction of the P30 column of wild-type TGF-α was measured in terms of EGF equivalents. The dilution curve had a slope that was parallel to that of the EGF standard. This value was also used to measure the colony-forming activity of the partially purified wild-type TGF-α (with EGF as a standard in the assay). The colony-forming activity of the partially purified wild-type TGF-α corre-

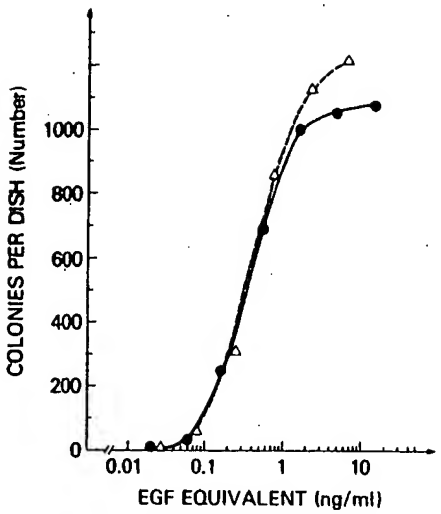

FIG. 4. Correlation between the activities in the binding and colony-forming assay for the partially purified wild-type TGF-α secreted by *S. cerevisiae*. The activity in the radioreceptor assay of the peak fraction from the P30 column was determined in EGF equivalent concentration. The value obtained was used for the soft-agar assay. Colonies of >62 μm (△) and the EGF standard (●) are shown.

sponded exactly to that of EGF (Fig. 4). Thus, we have partially purified a wild-type 50-amino-acid TGF-α showing the expected binding and colony-forming activities, which provides a reference substance for mutant TGF-αs that might show a dissociation of binding and colony-forming abilities.

Biological and biochemical activities of the partially purified TGF-α mutant proteins. Mutated TGF-αs were expressed by using the yeast system and partially purified on Bio-Gel P30 columns as described in Materials and Methods. Mutant TGF-αs were usually obtained from two different clones of yeast transformants. The CNBr-cleaved samples were purified through different Bio-Gel P30 columns for each mutant protein to avoid any possible contamination from one peptide to another. The purification profiles observed with the mutant TGF-αs were similar to those obtained for the wild-type TGF-α. Aliquots of the P30 column fractions were tested in radioreceptor and soft-agar assays. For all mutant proteins, the highest activity in both assays was always found in the same fraction of the Bio-Gel P30 column effluent (peak fraction). Extensive purification of a series of mutant proteins for screening purposes is not practical. Therefore, we needed a quantitation system that would allow us to compare mutant proteins with each other. Thus, the amount of TGF-α present in the peak fraction was estimated by radioimmunoassay with an antiserum to native TGF-α (obtained from W. Hargreaves), under denaturing conditions, as described in Materials and Methods. All values given in Table 1 were obtained from the peak fraction.

The controls done with the wild-type TGF-α showed (Fig. 4; Table 1) that binding and transforming activity were equivalent. The yeast vector without a TGF-α insert did not secrete any EGF-like proteins, as determined by both radioreceptor and soft-agar assay.

Two types of results were obtained upon assay of mutant proteins having different amino acid substitutions at Asp-47. In both [Ala-47]–TGF-α and [Asn-47]–TGF-α, binding ability was retained. Soft-agar and radioreceptor activities correlated for [Asn-47]–TGF-α; there was a lower value for

TABLE 1. Biological and biochemical activities of mutant TGF-α proteins secreted by *S. cerevisiae* and partially purified

Insert in the yeast expression vector	EGF equivalence (ng/ml) in:		Amt of TGF-α (ng/ml) in radioimmunoassay
	Radioreceptor assay	Soft-agar assay	
Wild-type TGF-α	700	700	2,000
	400	300	ND[a]
None	0	0	0
[Ala-47]–TGF-α	100	44	220
	66	48	ND
[Asn-47]–TGF-α	80	72	180
	75	72	525
[Glu-47]–TGF-α	3	3	42
[Ser-47]–TGF-α	10	4	60
[Ala-48]–TGF-α	0	0	16
	0	0	220
[Ile-48]–TGF-α	4	12	470
	2	7	490
[Met-48]–TGF-α	2	8	453
	0.5	2	420

[a] ND, Not determined.

colony-forming activity than for EGF-binding competition for [Ala-47]–TGF-α. [Ser-47]–TGF-α and [Glu-47]–TGF-α appeared to have lower activities in both assays than either wild-type TGF-α or [Ala-47]–TGF-α and [Asn-47]–TGF-α. These results indicate that neither the carboxyl charge nor the polarity of Asp-47 is essential for biological activity.

The effects of mutation of Leu-48, one of the 11 amino acids perfectly conserved among all the EGFs, TGF-αs, and viral EGF-like proteins, are dramatic. [Ala-48]–TGF-α totally lacked binding and colony-forming activity. [Ile-48]–TGF-α and [Met-48]–TGF-α had very little biological activity compared with wild-type TGF-α. Another substitution, [Met-48]–TGF-α, resulted in a truncated mutant lacking the last 2 amino acids and having a substitution of Leu to homoserine at position 48 following treatment with CNBr. Alternatively, if [Met-48]–TGF-α was not treated with CNBr, fusion proteins of TGF-α (mutated to Met in position 48) with 8 or 92 amino acids attached at the N terminus were obtained. Very low activities in binding and soft-agar assays were found for these mutants, whether or not they were cleaved with CNBr. Experiments on EGF and TGF-α have shown that an N-terminal extension does not markedly modify EGF-binding activity (12, 26). Therefore, the loss of activity obtained with [Met-48]–TGF-α that has not been CNBr treated was probably due to the mutation itself and not to the N-terminally extended fusion protein. We do not know whether the loss of activity observed with the TGF-α shortened to 48 amino acids and having a substitution of Leu-48 to homoserine is due only to the mutation or also to the lack of the last 2 amino acids.

The data obtained by radioimmunoassay on the partially purified wild-type and mutant TGF-α show that the amount of TGF-α detected was always higher than the amount determined by measurement of biological activity. This may be due to the presence in the fraction of a certain percentage of incorrectly folded TGF-α that might be recognized in a

radioimmunoassay under denaturing conditions but would not be biologically active. None of the mutant proteins seemed to be present in amounts equivalent to those observed for wild-type TGF-α in the partially purified fractions (whether radioimmunoassay, radioreceptor, or soft-agar assay was used for quantitation). It is not clear whether consistently less TGF-α was produced by the mutant constructs than by the wild type or whether the secreted mutant proteins were simply less well recognized by the antibody. Because of these uncertainties, the biological activities of the different mutant proteins cannot be accurately related to a known amount of mutant TGF-α protein. Even though radioimmunoassay should be used with caution for a quantitative evaluation of mutant TGF-α proteins, a positive reaction demonstrates that immunoreactive TGF-α was present in the P30 peak fraction for each mutant. Therefore, the fact that one of the mutant proteins ([Ala-48]–TGF-α) is biologically inactive can be attributed to the mutation itself, and not to the lack of production of the mutant protein by the yeast or its loss through purification. However, if the mutant proteins are in fact as immunoreactive as the wild type, then [Ala-47]–TGF-α and [Asn-47]–TGF-α are as active as wild-type TGF-α and [Glu-47]–TGF-α and [Ser-47]–TGF-α are less active; in contrast, [Ile-48]–TGF-α and [Met-48]–TGF-α are almost inactive. The differences between mutation of Asp-47 and Leu-48 would then be even more striking.

DISCUSSION

TGF-α shows sequence homologies with EGF, and both growth factors share the same cellular receptors (20). Even though EGF was discovered 25 years ago (7) and its properties have been extensively studied over the years (5), the binding site of EGF to its receptor has still not been determined, and the relationship between structure and function of EGF/TGF-α is still to be discovered. Particularly, we do not know whether binding to the receptor and signal transduction occur through one or more domains of the molecule or through which amino acids. We approached the question by performing site-directed mutagenesis of TGF-α and focused our attention on two adjacent amino acids, Asp-47 and Leu-48, located in the carboxy terminus and highly conserved in the EGF-like family of peptides. Unexpectedly, these two amino acids showed very different sensitivities to mutation and particularly to a substitution to Ala: [Ala-47]–TGF-α retained binding and colony-forming activities, whereas [Ala-48]–TGF-α completely lost both activities. These data show that Asp-47 and Leu-48 play very different roles in defining the structure and/or the activity of TGF-α. The other mutations performed on Asp-47 were substitutions to Asn, Ser, and Glu. [Asn-47]–TGF-α, like [Ala-47]–TGF-α, was active in binding and induction of colony formation, but [Ser-47]–TGF-α and [Glu-47]–TGF-α showed weaker growth factor activities. These results indicate that neither the carboxyl charge nor the polarity of Asp-47 is essential for biological activity. Interestingly, two of the EGF-like viral proteins, myxoma growth factor and Shope fibroma growth factor (6, 30), have Asn instead of Asp in position 47; we have shown that [Asn-47]–TGF-α retains biological activity.

Substitution of Leu-48 to Met and Ile led to mutant proteins with very low activities, whereas substitution to Ala led to complete loss of activity. We did not expect that a mutation of Leu to Ile (which have similar sizes and polarities) would cause such a strong effect. Thus, Leu-48, which is conserved perfectly among all the EGF-like peptides, seems to be essential, through its exact geometry, for the biological activity of TGF-α.

The mutant proteins tested so far, when active, showed parallel behaviors in binding and colony formation. Some mutant proteins lost all activities, and we assume that the binding capacity has been lost. We have not been able to dissociate the binding and colony-forming abilities by using any of the present series of mutant proteins, and it is necessary to screen more of them in search of an antagonist of TGF-α.

Results relating to the biological activity of EGF show that derivatives of mouse EGF and human EGF (EGF 1-47) lacking the carboxy-terminal 6 amino acids as a result of enzymatic digestion are less potent than the intact molecule in mitogenic stimulation of fibroblasts, but retain full biological activity in in vivo assays (inhibition of gastric acid secretion) (16). On the other hand, naturally occurring truncated forms of rat EGF, which lack the carboxy-terminal 5 amino acids (rEGF 2-48) are as potent as mouse EGF (mEGF 1-53) in receptor-binding and mitogenic assays (27). We do not know whether the discrepancies observed are due to the origin of the molecule (artificial or natural) or to the type of bioassay used. In any event, all of these EGF-related molecules, which are shorter than mouse or human EGF, still retain Leu-47. We have shown that in TGF-α, the corresponding residue, Leu-48, is critical for the biological activity.

Recent data on the three-dimensional structure of mouse EGF obtained by nuclear magnetic resonance show that even though Asp-46 and Leu-47 (Asp-47 and Leu-48 in TGF-α) are both solvent accessible (8, 22, 22a), their side chains point in opposite directions in the beta-sheet structure. Therefore, the role of these adjacent amino acids in the structure and, consequently, the function of EGF might be very different. Our data show that the amino acids Asp-47 and Leu-48 of TGF-α are not equally important for the biological activity of TGF-α, despite their conservation among the EGF-like peptides. From the dramatic loss in biological activity which is characteristic of mutation of Leu-48, we also suggest that this residue is involved in binding to the cellular receptors either by direct interaction with the receptor or by providing the proper conformation to the molecule.

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Functional Analysis of Conserved Histidines in ADP-Glucose Pyrophosphorylase from *Escherichia coli*[1]

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Two absolutely conserved histidines and a third highly conserved histidine are noted in 11 bacterial and plant ADP-glucose pyrophosphorylases. These histidines were individually mutagenized in the *E. coli* enzyme to glutamine in order to determine their function. Glutamine mutations at residues 143 and 156 produced functional enzymes in cell extracts with slightly lower than wild-type specific catalytic activities and with same heat stability characteristics of the wild-type enzyme. Substitution of residue 83 with glutamine however produced an enzyme having decreased thermal stability. Additional mutageneses at residue 83 with asparagine, arginine, or aspartate gave rise to enzymes having a progressively decreasing trend in thermal stability. These mutants are more susceptible to proteolysis than wild-type enzyme. Kinetic analysis of H83Q and H83N indicates that histidine 83 is not involved in the catalytic mechanism or in substrate binding but possibly in maintenance of the active catalytic structure. © 1998 Academic Press

ADP-glucose pyrophosphorylase (ATP:a-glucose-1-phosphate adenylyltransferase, EC 2.7.7.27) is an example of an allosterically regulated enzyme whose general function is conserved in evolutionarily widely separated organisms. Bacterial and higher plant enzymes catalyze the reversible reaction between ATP and glucose 1-phosphate to yield ADP-glucose and pyrophosphate. Metabolically, this represents the first step in glycogen and starch biosynthesis in bacteria and plants, respectively. Allosteric regulation by glucose metabolites modulates enzyme activity; however, the structural specificity of the allosteric modifiers is different for the bacterial and plant enzymes, reflecting their differing assimilating glucose metabolic pathways.

Details of the mechanisms of catalysis and regulation for this enzyme provide insight into general principles of protein evolution of structure. Although tertiary structure information is presently unavailable for this enzyme, primary sequence data has been collected from a large array of bacterial, cyanobacterial, and higher plant pyrophosphorylases (1). Comparisons of these sequences highlight those strictly conserved residues whose functions are essential. Elucidation of their specific roles in enzyme function have been used by site-directed mutagenesis techniques. This study initiates studies on the structure-function roles of histidine residues for the *E.coli* ADP-glucose pyrophosphorylase.

Histidines were chosen as targets for mutagenesis studies due to their potential role in catalytic mechanisms involving acid-base catalysis. Other roles for histidines in substrate binding and/or in protein structure stabilization are possible as well. Two histidines are absolutely conserved in every ADP-glucose pyrophosphorylase sequenced to date. A third histidine is highly conserved in nine out of eleven sequences; the remaining two exist as glutamine. Site directed mutagenesis studies were carried out to replace each of these histidines with glutamine initially; additional residues asparagine, arginine and aspartate were substituted at the 83 position to further test its function.

EXPERIMENTAL PROCEDURES

Reagents. [^{32}P]pyrophosphate, [^{14}C]glucose-1-phosphate and [^{35}S]-dATP were purchased from DuPont-New England Nuclear. Enzymes for DNA manipulation and sequencing were from New England Biolabs or Boehringer Mannheim. Oligonucleotides were synthesized and purified by the Macromolecular Facility at Michigan State University. All other reagents were purchased as the highest quality available.

Bacterial strains and media. Bacterial strains used included *E. coli* MV1193 (Δ(lac-proAB) rpsL thi endA spcB15 hsdR4 Δ(srl-recA)306::Tn 10(tetr) F'[traD36 proAB+ lacIq lacZΔM15]), E. coli CJ236 (dut, ung, thi, rel A/pCj105 (Cmr)), and *E. coli* K12 G6MD3 (Hfr, his thi, Strr, Δ(mal - asd)).

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His-83 5'-TG GTG CAG CAC ATT CAG CG-3'
3'-AC CAC GTC GTG TAA GTG GC-5'

Gln-83 3'-AC CAC GTC GTT TAA GTG GC-5'
*

Asn-83 3'-AC CAC GTC TTG TAA GTG GC-5'
*

Arg-83 3'-AC CAC GTC GCG TAA GTG GC-5'
*

Asp-83 3'-AC CAC GTC CTG TAA GTG GC-5'
*

His-156 5'-TT ATC GAT CAC GTC GAA AA-3'
3'-AA TAG CTA GTG CAG CTT TT-5'

Gln-156 3'-AA TAG CTA GTT CAG CTT TT-5'
*

His-143 5'-CG GGC GAC CAT ATC TAC AA-3'
3'-GC CCG CTG GTA TAG ATG TT-5'

Gln-143 3'-GC CCG CTG GTT TAG ATG TT-5'
*

FIG. 1. Nucleotide sequences and encoded protein sequences of the ADP-glucose pyrophosphorylase gene in the regions of His-83, His-143, and His-156, and the synthetic oligonucleotides used to generate amino acid substitutions at these positions. The base substitutions are marked with asterisks.

G6MD3 cells were grown in enriched medium which contained 1.1% K_2HPO4, 0.85% KH_2PO4, 0.6% yeast extract, 0.2% glucose, pH 7.0. G6MD3 cell cultures also contained 50 mg/ml diaminopimelic acid, required for growth. MV1193 cells were grown in two media: M9 minimal medium containing 0.56% Na_2HPO4, 0.3% KH_2PO4, 0.05% NaCl, 0.1% NH_4Cl, 0.1 mM $CaCl_2$, 1 mM $MgSO_4$, 0.001% thiamine, and 0.2% glucose, and LB medium containing 1% tryptone, 0.5% yeast extract, 0.5% NaCl. CJ236 cells were grown in LB medium containing 30 mg/ml chloramphenicol.

Site-directed mutagenesis. The gene for the native *E. coli* K12 ADP-glucose pyrophosphorylase was subcloned from the pOP12 plasmid (2) into M13mp18RF. The fragment used, the 1.9 kilobase *Hinc* II fragment of pOP12, contains the complete coding region for ADP-glucose pyrophosphorylase as well as the 474 base upstream region which encodes promoter site(s) necessary for expression of the gene(3). Site-directed mutagenesis was performed using the method of Kunkel (4,5). The mutant oligonucleotides used are shown in Fig. 1. All mutant DNAs were completely sequenced.

TABLE I

Specific Catalytic Activities in Crude Cell Extracts

	Specific activity (units/mg protein)
wild-type	5.40 ± 0.10
H156Q	1.43 ± 0.06
H143Q	2.69 ± 0.04
H83Q	3.70 ± 0.10
H83N	2.15 ± 0.05
H83R	0.15 ± 0.01
H83D	0.026 ± 0.002

Note. Reactions were performed at 37 °C as described under "Experimental Procedures". Data represent the averages of two identical experiments ± the average difference of the duplicates.

[a] One unit of enzyme activity is expressed as the amount of enzyme required to form one micromole of ATP per minute at 37 °C assayed in the pyrophosphorolysis direction as described under "Experimental Procedures."

Expression and purification of mutant and wild-type enzymes. For expression of wild-type and mutant enzymes, G6MD3 cells were infected with M13 phage carrying the desired gene.

Wild-type enzyme was purified according to established procedures (6, 7). Mutant enzymes, H83Q and H83N, were purified using these same procedures with the following modifications, found to prevent denaturation of these more labile proteins. The 60 °C heat step was replaced with a 35% ammonium sulfate saturation step in which enzyme remained in solution. This was followed by a 55% ammonium sulfate saturation step in which enzyme precipitated. Mutant enzyme was resuspended in 0.05 M glycylglycine, 1 mM EDTA, 5 mM DTT, pH 7.0 and dialyzed versus the same at 4 °C. This mutant enzyme sample was chromatographed on DEAE-Sepharose as de-

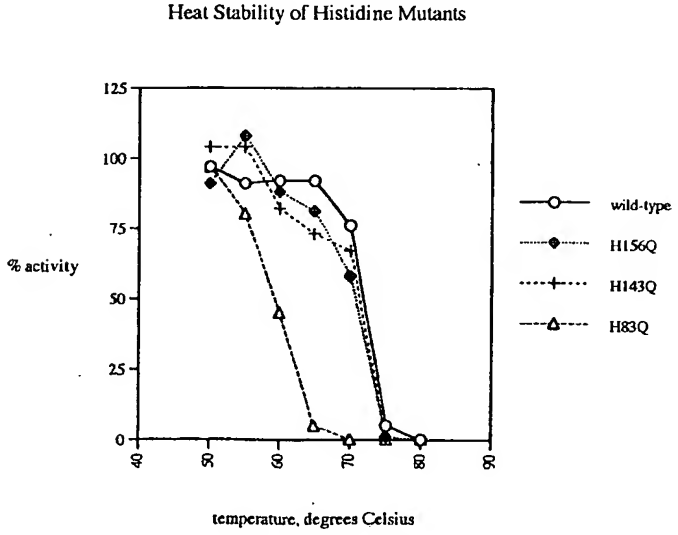

FIG. 2. Thermal stability comparison of wild-type, H156Q, H143Q, and H83Q proteins. Individual samples of G6MD3 cell extracts were heated simultaneously at the indicated temperatures for five minutes in the presence of 30 mM sodium phosphate, pH 7.0. After cooling on ice, samples were clarified by centrifugation and supernatants were assayed in the synthesis direction of assay. Symbols represent wild-type (O), H156Q (♦), H143Q (+), and H83Q (Δ).

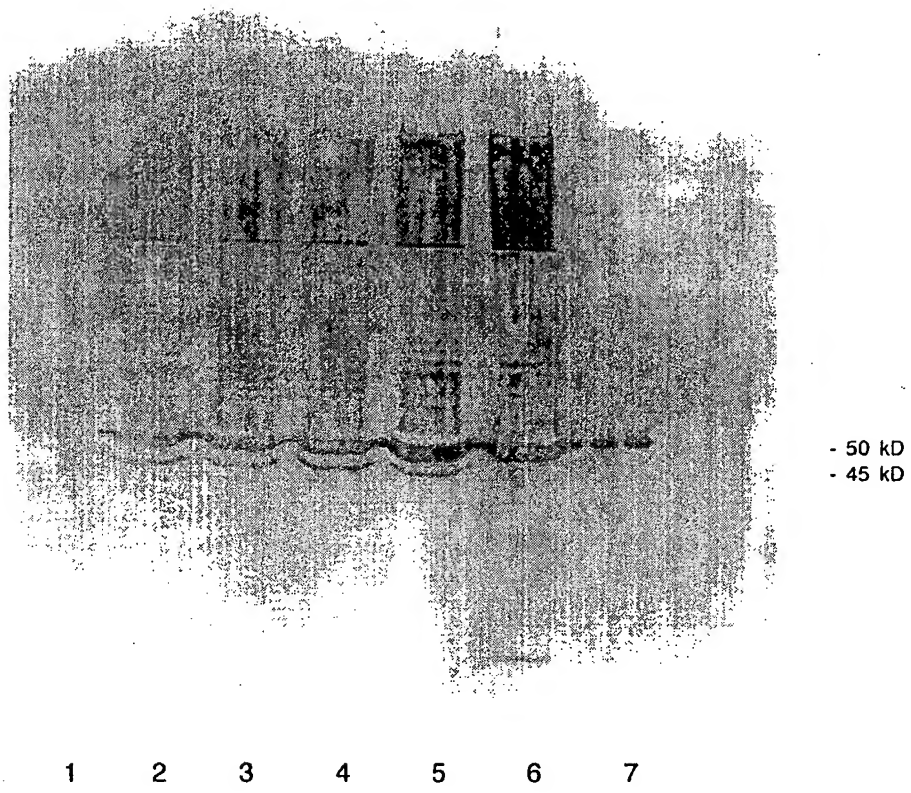

FIG. 3. Western Blot comparison of wild-type and mutant ADP-glucose pyrophosphoryase expression in G6MD3 cells. Extracts of expressed cells were subjected to SDS-PAGE; in each case 8 mg of protein was applied to the gel. Proteins were transferred to nitrocellulose and visualized using antiserum prepared against *E. coli* ADP-glucose pyrophosphorylase as described under "Experimental Procedures". Lane1 represents blank G6MD3 cells in which no ADP-glucose pyrophosphoryase was expressed. Lanes 2 - 6 represent expressed H83D, H83R, H83N, H83Q, and wild-type enzymes, respectively. Lane 7 represents purified wild-type ADP-glucose pyrophosphorylase.

scribed (6, 7). Following DEAE-Sepharose chromatography, the enzyme was concentrated and dialyzed as described, and then chromatographed directly on a Mono Q HR 5/5 Fast Protein Liquid Chromatography column. Purified mutant enzymes were desalted using an Econopak 10 desalting column (BioRad) equilibrated with 0.05 M Tris-HCl pH 7.5, 1mM EDTA, 0.5 mM DTT, 10 % glycerol. KCl was added immediately to a concentration of 1 mM and the enzyme quick-frozen in liquid nitrogen and stored at −80 °C. The Green A column was omitted during both mutant enzyme purifications. Each purified enzyme migrated as a single band (MW 50 kD) on SDS-PAGE.

Preparation of cell extracts for assay. Crude cell extracts of wild-type and mutant enzymes were prepared by resuspension of frozen, infected G6MD3 cells (0.5 grams) in 10 ml 0.05 M glycylglycine, 1 mM EDTA, 5 mM DTT, pH 7.0, on ice. Cells were disrupted by sonication using time and energy settings which were empirically found to be the minimum for complete cell disruption under these conditions. Cell extracts were clarified by centrifugation at 4 °C and assayed the same day of their preparation in all cases.

Enzyme kinetics. Enzymatic activity in the ADP-glucose synthesis direction at 37 °C was measured according to the method of Preiss, et al. (8). For assay of wild-type and mutant enzymes, reaction mixtures (final volume 200 ml) contained 0.1 mmol of [^{14}C] glucose-1-phosphate (specific activity 500 -1000 cpm/nmol), 0.3 mmol of ATP, 1.0 mmol of $MgCl_2$, 0.3 mmol of fructose-1,6-bisphosphate, 20 mmol of Hepes buffer, pH 7.0, and 100 mg of bovine serum albumin.

Enzymatic activity in the pyrophosphorylase direction at 37 °C was measured according to Preiss, et al. (8). Wild-type and mutant reaction mixtures (final volume 260 ml) contained 1.0 mmol of ADP-glucose, 0.43 mmol of [^{32}P]pyrophosphate (specific activity 4000 cpm/nmol), 2.0 mmol of $MgCl_2$, 0.78 mmol of fructose 1,6-bisphosphate, 10.0 mmol of Tris-HCl, pH 8.5, and 100 mg of bovine serum albumin.

Kinetic characterization. Kinetic data were plotted as intial velocity versus substrate or effector concentration. Data were replotted as double-reciprocal plots and the method of Wilkinson (9) was used to determine V_{max}. Sigmoidal plots were replotted as Hill plots to obtain kinetic constants. For sigmoidal data the following expressions for kinetic constants were used: $A_{0.5}$, $I_{0.5}$, and $S_{0.5}$, concentration of activator, inhibitor, or substrate, respectively, giving 50% maximal activation, inhibition, or maximal velocity. Duplicates were run in each case; kinetic constants are expressed as the mean ± the difference from duplicate determinations.

Thermal stability. Crude cell extracts were prepared as described above. Potassium phosphate, pH 7.0, was added to give a final concentration of 30 mM phosphate. Individual samples (50 μl volume) of crude extracts were heated for 5 min in a water bath equilibrated at the specified temperature, then immediately placed on ice. Samples were centrifuged, then assayed in the synthesis direction as described above.

Protein determination. Protein was assayed by the method of Smith, et al. (10) using bovine serum albumin as the standard.

SDS PAGE and Western blotting. SDS PAGE was performed in 10% gels using the method of Laemmli (11). Following electrophoresis, proteins were transferred to nitrocellulose membranes (12).

Staining was accomplished using rabbit antibody raised against the E coli ADP-glucose pyrophosphorylase, followed by alkaline phosphatase-labelled goat anti-rabbit antibody and subsequent visualization.

RESULTS

None of the three conserved histidines, H83, H143, or H156, are essential for catalytic activity in the *E. coli* enzyme. In each case when glutamine is substituted for histidine a functional enzyme results, though the activity is slightly lower than wild-type (Table I). An interesting, and quite critical observation of the H83Q mutant was that the original sonication conditions resulted in low specific activity measurements. These conditions used extended times of sonication (five 15 second pulses) at a high intensity level while keeping the sample on ice. It was found that a shorter period of sonication (two fifteen second pulses) at a low intensity setting (with sample on ice) resulted in crude extracts with wild-type levels of activity. Analysis of the heat stabilities of these mutants (Figure 2) illustrates the probable cause for the disparate H83Q specific activities; this mutant is heat labile in comparison to the wild-type enzyme or the other two histidine mutants. The initial low activity presumably resulted from denaturation during sonication.

Three additional substitutions at position 83 were prepared and analyzed: asparagine, arginine, and aspartate. Specific catalytic activities for these mutant enzymes in crude cell suspensions (prepared using minimum sonication conditions) indicate that asparagine is also a conservative change, but that arginine and aspartate are deleterious to enzyme function (Table I). Cell extracts used for catalytic assay were subjected to SDS PAGE and western blotting to assess whether the low specific activities were the result of low protein expression in the original host cultures (Figure 3). The western blot reveals that significant protein is expressed in each case, discounting lack of expression as a reason for the low specific activity. The western blot also reveals an antibody-stained protein band which migrates as a 45 kD fragment, in addition to the native enzyme band at 50 kD. This 45 kD band is present in all of the mutant lanes but is absent in the wild-type sample. This suggests that proteolytic processing is a factor in the mutant expressions.

Heat stability decreases as the substitution at position 83 progresses from histidine to glutamine to asparagine to arginine as shown in Figure 4. The aspartate mutant activity was too low to measure heat stability.

The two most heat stable mutants, H83Q and H83N, were purified, initially using procedures developed for purification of the wild-type enzyme. During the purifications difficulties were encountered in maintaining the stabilities of both these mutant enzymes. In particular, mutants were found to precipitate under conditions of low salt or low protein concentration, especially

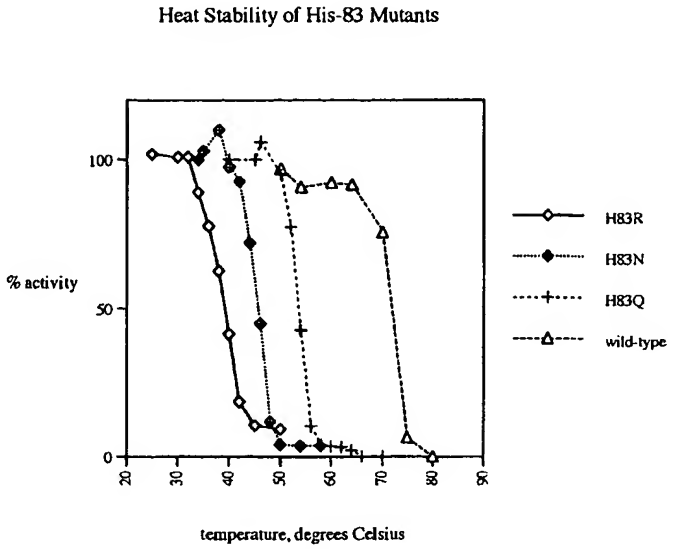

FIG. 4. Thermal stability comparison of wild-type, H83Q, H83N, and H83R proteins. Individual samples of G6MD3 cell extracts were heated at the indicated temperatures for five minutes in the presence of 30 mM sodium phosphate, pH 7.0. After cooling on ice, samples were clarified by centrifugation and supernatants were assayed in the synthesis direction of assay. Symbols represent wild-type (Δ), H83Q (+), H83N (♦), and H83R (◇).

during hours of dialysis following Fast Protein Liquid Chromatography on Mono Q. KCl at a final concentration of 1 mM was found to reverse or prevent this precipitation problem. H83Q and H83N purifications were repeated using a desalting column in place of dialysis at the final step of protein transfer into its storage buffer, to which was added 1 mM KCl. Despite these efforts to preserve mutant protein integrity, the specific catalytic activities of the two purified mutants were somewhat low in comparison to that of the wild-type enzyme: 18 and 28 units per mg for the asparagine and glutamine mutants, respectively, versus 74 units per mg for the wild-type enzyme (Table II).

It was also observed that the same purified enzyme sample (glutamine or asparagine mutant) showed a gradual loss of specific activity upon repeated freezings and thawings. Wild-type enzyme is resistant to this kind of activity loss.

Kinetic constants were measured for enzymes in the synthesis direction of assay, the metabolically significant direction. Kinetic constants (Table II) for substrates glucose 1-phosphate, ATP, and cofactor Mg^{2+} are similar in wild-type and mutant enzymes; a less than two-fold difference was obtained for each of these ligands. Similarly, the inhibitor AMP kinetic constant is unchanged as a result of histidine 83 substitution. Only the activator, fructose-1,6-bisphosphate, shows a large change in kinetic constant measurement ; $A_{0.5}$ values for both glutamine and asparagine mutants are on the order of 7-fold greater than the wild-type $A_{0.5}$.

TABLE II

Kinetic Constants of Wild-Type and Mutant ADP-Glucose Pyrophosphorylase

Glucose 1-phosphate	Wild-type	H83Q	H83N
Km (μM)	27.1 ± 0.9	22.8 ± 9.3	29.2 ± 4.3
ATP $S_{0.5}$ (μM)	334 ± 14	567 ± 5	535 ± 57
$MgCl_2$ $S_{0.5}$ (mM)	2.68 ± 0.09	4.56 ± 0.13	4.09 ± 0.08
AMP $I_{0.5}$ (μM)	89.5 ± 4.5	86.5 ± 14.5	90.8 ± 10.2
Fru 1,6-bis-P $A_{0.5}$ (μM)	33 ± 7	240 ± 32	178 ± 22
V_{max} (units/mg)	74.0 ± 9.0	28.4 ± 0.3	18.0 ± 1.1

Note. Reactions were performed at 37 °C in the synthesis direction of assay as described under "Experimental Procedures". Data represent the average of two identical experiments ± the average difference of the duplicates.

DISCUSSION

No absolutely conserved histidine is essential for catalysis in the *E. coli* ADP-glucose pyrophosphorylase enzyme. In fact, of the three conserved histidines, two may be replaced by glutamine with little change in specific catalytic activity (as measured in crude cell suspensions), and no change in heat stability. These residues, at positions 143 and 156 in the *E. coli* enzyme, must not function in the catalytic mechanism, nor be essential to protein structure stabilization.

Histidine 83 is also not essential for catalysis. Conservative replacements by glutamine or asparagine do not affect specific catalytic activity, thereby ruling out the possibility of His-83 involvement in catalysis. Furthermore, these substitutions have negligible effect on substrate, cofactor, and inhibitor binding, as judged by the similarities between wild-type and mutant kinetic constants measured for these ligands. However, perturbation of the activator (fructose 1,6-bis-phosphate) binding site constant does occur with these substitutions.

Stabilization of protein tertiary structure is concluded to be the reason for histidine conservation at the 83 position. The decrease in thermal stability and the increase in proteolytic susceptibility for the mutants provides support for this conclusion. Several studies suggest that thermal stability is a key determinant of proteolytic susceptiblity in the cell (13). Certainly, proteases show a preference for denatured proteins *in vitro* (14).

In light of this, it is not surprising that the "conservative" mutants H83Q and H83N were difficult to isolate in stable form and that precipitation and loss of activity ocurred during the purification process. The specific catalytic activities measured for these purified forms can be considered to be minimum values until purification conditions are found during which mutant stabilities can be maintained.

It is not possible to conclude whether His-83 is directly involved in fructose 1,6-bis-phosphate (FBP) binding. Previous covalent modification and site-directed mutagenesis studies identified residues at the N-terminus of the bacterial enzyme which are involved in FBP binding (15,16,17). Since His-83 is also located in this region it would not be surprising if it were to be part of the FBP site. However, perturbation of the FBP site in His-83 mutants may simply be a secondary effect from localized destabilization of tertiary structure in the FBP binding domain by a distant substituted residue 83. This would be reasonable given the effect of His-83 mutations on overall protein stability. It is also difficult to rationalize why an amino acid involved in bacterial enzyme FBP binding would be so well conserved in organisms which have evolved a different activator binding specificity. His-83 may therefore be conserved solely to preserve the folded, active state of the protein.

Efforts are continuing to probe the functional roles of other absolutely conserved amino acids in this enzyme. Recent success in the growth of crystals of E. coli enzyme suitable for x-ray diffraction will make it possible to evaluate whether His-83 does or does not participate directly in FBP binding.

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